Prevalence and mutation analysis of the spike protein in feline enteric coronavirus and feline infectious peritonitis detected in household and shelter cats in western Canada

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Abstract

Feline infectious peritonitis (FIP) is a fatal disease for which no simple antemortem diagnostic assay is available. A new polymerase chain reaction (PCR) test has recently been developed that targets the spike protein region of the FIP virus (FIPV) and can identify specific mutations (M1030L or S1032A), the presence of which indicates a shift from feline enteric coronavirus (FeCV) to FIPV. This test will only be useful in the geographical region of interest, however, if the FIP viruses contain these mutations. The primary objective of this study was to determine the presence of the M1030L or S1032A mutations in FeCV derived from stool samples from a selected group of healthy cats from households and shelters and determine how many of these cats excrete FeCV. The secondary objective was to evaluate how often these specific FIPV mutations were present in tissue samples derived from cats diagnosed with FIP at postmortem examination. Feline enteric coronavirus (FeCV) was detected in 46% of fecal samples (86/185), all were FeCV type 1, with no difference between household or shelter cats. Only 45% of the FIPV analyzed contained the previously reported M1030L or S1032A mutations. It should be noted that, as the pathological tissue samples were opportunistically obtained and not specifically obtained for PCR testing, caution is warranted in interpreting these data.

Résumé

La péritonite infectieuse féline (FIP) est une maladie fatale pour laquelle il n'existe pas de test diagnostique ante-mortem simple. Une nouvelle épreuve d'amplification en chaîne par la polymérase (PCR) a récemment été développée et qui vise la région de la protéine de spicule du virus FIP (FIPV) et peut identifier les mutations spécifiques (M1030L ou S1032A), la présence desquelles indique un glissement du coronavirus entérique félin (FeCV) vers le FIPV. Cette épreuve sera utile uniquement dans la région géographique d'intérêt, toutefois, si les virus FIP ont ces mutations. L'objectif premier de la présente étude était de déterminer la présence des mutations M1030L ou S1032A chez FeCV obtenu d'échantillons de fèces provenant d'un groupe sélectionné de chats en santé issus de maisonnée et refuges et de déterminer combien de ces chats excrètent FeCV. L'objectif secondaire était d'évaluer à quelle fréquence ces mutations spécifiques de FIPV étaient présentes dans des échantillons de tissu provenant de chats diagnostiqués avec FIP lors d'examen post-mortem. Le FeCV fut détecté dans 46 % des échantillons fécaux (86/185), tous de type FeCV 1, et aucune différence notée entre les chats de maisonnée ou de refuge. Seulement 45 % des FIPV analysés contenaient les mutations M1030L ou S1032A rapportées précédemment. Il faut noter que, étant donné que les échantillons de tissus pathologiques furent obtenus de manière opportuniste et non spécifiquement obtenus pour analyse par PCR, l'interprétation des résultats est à faire avec précaution.

(Traduit par Docteur Serge Messier)

Introduction

Feline infectious peritonitis (FIP) is a fatal, multi-systemic disease for which no treatment or effective vaccine is currently available (1). This disease can occur in cats following fecal-oral infection with feline enteric coronavirus (FeCV). An infection with FeCV will usually only result in mild clinical gastrointestinal signs. In approximately 5% of cats infected with FeCV, however, this virus mutates into an FIP-causing virus (FIPV) (2). It should be noted that 2 different FeCV serotypes, types 1 and 2, circulate in the feline population (3). Type 1 is most prevalent in naturally infected populations, whereas FeCV type 2 has emerged as a recombination of canine and feline coronaviruses and is relatively rare. Both serotypes can mutate and cause FIP (4).

In a single cat, FeCV and FIPV are genetically very closely related and only a few mutations are detectable when the enterocyte-infecting FeCV is compared to the monocyte- and macrophage-infecting FIPV. While the exact genetic change that mediates the increase in pathogenicity is unknown, there is strong evidence that specific mutations in the spike-protein (S) coding region play a major role in this transition

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(5–7). M1030L or S1032A mutations in the S-protein genomic region of this coronavirus have been identified in viruses from tissues of > 95% of FIP cats that were examined in Europe (5,6).

The clinical diagnosis of FIP is complex, especially in the early stages of the disease. While histopathology combined with immunohistochemistry to detect FeCV antigens is the gold standard, this requires tissue obtained through biopsy or necropsy (8). Polymerase chain reaction (PCR) on blood or aspirates is an attractive alternative for diagnosing infectious diseases. A new PCR has recently been developed that could be a major diagnostic improvement (5,9). The primary objective of this study was to determine if the FIPV-specific M1030L and/or S1032A mutations can also be detected in healthy cats by analyzing the spike protein in stool samples obtained from shelter and household cats. The secondary objective was to assess the presence of these mutations in the S-protein genomic region in coronaviruses of cats diagnosed with FIP at postmortem examination in Saskatchewan and Alberta.

Materials and methods

Samples

This protocol was approved by the University of Calgary Animal Care Committee (VSACC AC16-0268).

Fresh fecal samples were collected from 185 domestic and stray cats on intake to various shelters, veterinary clinics, and from private cat owners in Calgary. Of all fecal samples obtained, 130 were derived from cats that were offered to shelters and 55 were sampled in a veterinary clinic or at home by their owners. All cats were clinically healthy. With a conservative estimated prevalence of 33% to 50% in adult cats, we anticipated that this would result in 61 to 93 unique strains of FeCV (1,10).

We obtained 63 formalin-fixed, paraffin-embedded (FFPE) archived tissue samples, 4 frozen tissue samples, and 2 postmortem peritoneal fluid samples from the Diagnostic Services Unit at the University of Calgary Faculty of Veterinary Medicine and the Department of Pathology at the Western College of Veterinary Medicine. Tissue samples were selected on the basis of typical histopathological lesions that are indicative of FIPV infections (granulomatous inflammation and vasculitis, samples FIP 13, 14, and 17), preferably supported with a positive result on immunohistochemistry for the FeCV antigen (samples FIP 1 to 12 and 16 and 18). A few cases were included with only gross lesions as the basis of the FIP diagnosis (samples FIP 15, 19, and 20). Multiple tissue types could be present in a single paraffin block.

All samples came from a pool of spayed, neutered, or intact male and female cats, ranging in age from 3 mo to 21 y. Representative breeds included Siamese, Ragdoll, LaPerm, Persian, Himalayan, Sphynx, Russian blue, and Cornish rex, as well as a number of mixed breeds. Unfortunately, precise signalment was not available on many of the shelter cats, but they fell within the above-mentioned age range.

If known, the region, city, or quadrant of the city of Calgary where the samples were taken was included in the name of the sequences used for analysis, as indicated in Figures 2 and 3. All diagnosed FIP cases are indicated with 'FIP', whereas all positive fecal samples are indicated as 'FeCV'.

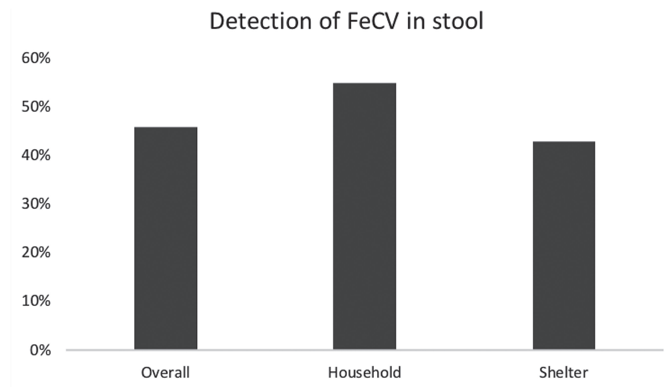

Figure 1. Detection of FeCV in stool samples from healthy cats using PCR in Calgary and surrounding areas.

RNA extraction

Viral ribonucleic acid (RNA) was extracted from the fecal samples using either an EZNA Universal Pathogen Kit (Omega Bio-tek, Norcross, Georgia, USA) or a Norgen Stool Viral RNA Kit (Norgen Biotek, Thorold, Ontario) as per manufacturer's instructions. The FFPE tissue was sectioned on a microtome and 3 to 5 10-μm scrolls were obtained from each sample. The Ambion RecoverAll Kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) was used to extract viral RNA from the FFPE scrolls as directed. Viral RNA from frozen tissue and peritoneal fluid was extracted using Trizol reagent (Sigma Aldrich Canada, Oakville, Ontario). All RNA was assessed for quantity and quality with a Nanodrop spectrometer (ThermoFisher Scientific) and frozen at −20°C.

PCR

Feces, FFPE tissue, peritoneal fluid, and frozen tissue were screened to identify animals positive for FeCV by using a nested reverse transcription polymerase chain reaction (RT-PCR) targeting the highly conserved 3′-untranslated region (3′UTR) of the viral genome (11). Gene-specific complementary deoxyribonucleic acid (DNA) was synthesized using Superscript Reverse Transcriptase II (Invitrogen, Carlsbad, California, USA) and the antisense primer U211R (see Table I) as per manufacturer's instructions. We conducted a nested PCR using Phusion High-Fidelity DNA Polymerase (Invitrogen) and specific primers (U211R and U204F) for the first reaction and specific primers (U276F and U205R) for the second reaction. PCR cycling conditions were 98°C for 30 s, followed by 35 cycles of 98°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with an additional final elongation step at 72°C for 10 min. The expected product size was 223 basepair (bp) for the first reaction and 177 bp for the second reaction.

Samples positive for 3′UTR were then reverse transcribed using Superscript Reverse Transcriptase II (Invitrogen) and the antisense primer S585R to produce gene-specific complementary DNA for the S protein region of interest. We conducted a nested PCR with cycling conditions identical to those previously described using specific primers (S585R and S866F) for the first reaction and specific primers (S876F and S1000R) for the second reaction. The expected product size was 598 bp for the first reaction and 142 bp for the second reaction (5).

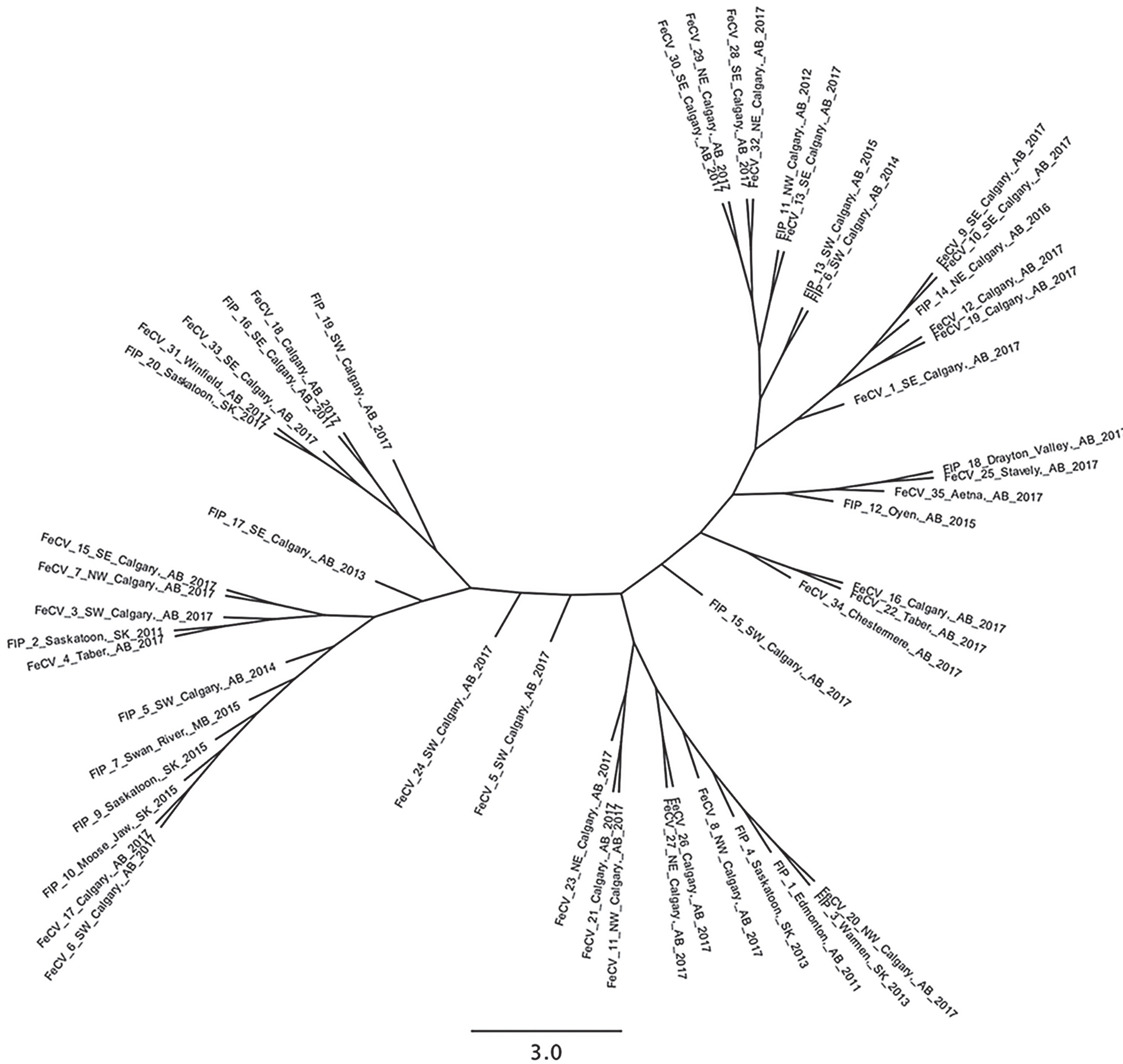

Figure 2. Unrooted nucleic acid maximum likelihood tree of the partial spike protein sequence of FeCV and FIPV as detected in western Canada.

Amplified products were separated on a 1% agarose gel by electrophoresis, visualized with Safeview (Applied Biological Materials, Richmond, British Columbia), and the bands were cut out for DNA extraction using an EZNA DNA Gel Extraction Kit (Omega Bio-tek) as per manufacturer's instructions. Extracted DNA was sent to the University of Calgary Core DNA Services in Calgary, Alberta or the TCAG DNA Sequencing Facility at Sick Kids Hospital in Toronto, Ontario for Sanger sequencing of the amplified products.

Phylogenetic analysis

The nucleotide fragments were translated into amino acid sequences in Geneious 10.2.3 (Biomatters, Auckland, New Zealand) and aligned with Clustal Omega (12). Amino acid sequence numbering was based on a type-1 feline coronavirus (GenBank accession number NC_002306). jModelTest (13) was used to determine the most appropriate evolutionary model for our data. The generalized time-reversible model was selected to construct a maximum likelihood phylogenetic tree using PhyML (14).

Statistical analysis

The occurrence of FeCV in household and shelter cats was analyzed using Chi-squared tests with $P < 0.05$ considered significant.

Results

Prevalence of FeCV in samples from Calgary and surrounding areas

Overall, 46% of cats sampled (86/185) tested positive for feline enteric coronavirus (FeCV) in their feces. The percentage of shelter

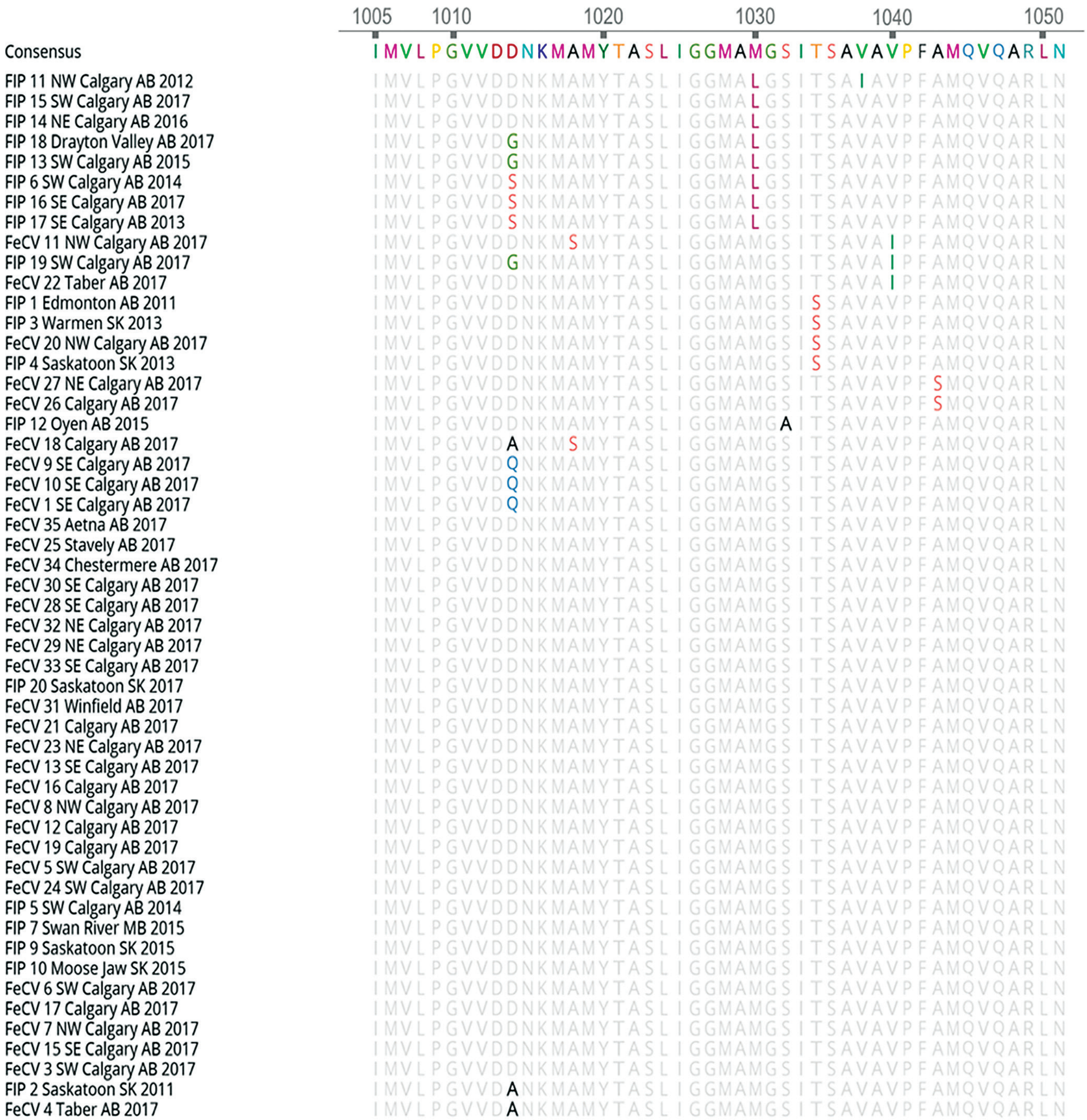

Figure 3. Amino acid sequence alignment of the partial FeCV and FIPV spike proteins. Agreements with the consensus are gray; disagreements with the consensus are highlighted. Numbering on top is based on the amino acid positions in the spike protein of the reference genome: GenBank accession number NC_002306.

Table I. Primers used for FeCV and FIPV detection and characterization.

Primer name	Reaction	Primer sequence	Product size
P204 Forward	UTR1	5′-CACTAGATCCAGACGTTAGCTC-3′	223 bp
P211 Reverse	UTR1	5′-GCTCTTCCATTGTTGGCTCGTC-3′	223 bp
P276 Forward	UTR2	5′-CCGAGGAATTACTGGTCATCGCG-3′	177 bp
P205 Reverse	UTR2	5′-GGCAACCCGATGTTTAAAACTGG-3′	177 bp
S866 Forward	S1	5′-CAATATTACAATGGCATAATGG-3′	598 bp
S585 Reverse	S1	5′-CCCTCGAGTCCCGCAGAAACCATACCTA-3′	598 bp
S877 Forward	S2	5′-GGCATAATGGTTTTACCTGGTG-3′	142 bp
S1000 Reverse	S2	5′-TAATTAAGCCTCGCCTGCACTT-3′	142 bp

cats that were FeCV-positive (43%, 56/130) did not differ from the percentage of positive household cats (55%, 30/55), X^2 (1, n = 185) = 2.0434, P = 1.58 (Figure 1). No difference was found between the percentage of FeCV-positive cats originating from different quadrants of the city and those from outside city limits, X^2 (5, n = 185) = 7.298, P = 0.199.

Phylogenetic analysis

All sequences were of the FeCV type 1. We found no difference between FeCV and FIPV samples, as the clustering and distribution appeared to be randomly dispersed in the phylogenetic tree. No clear transmission or relation between samples could be determined (Figure 2). Many of the FIPV and FeCV strains showed a very similar amino acid sequence (Figure 3). As most of the mutations observed in the nucleotide sequence did not result in an amino acid mutation (silent mutation), the variability in the amino acid sequence is limited and only the alignment is presented.

Mutation analysis

We were able to amplify the S-protein region of interest from 35/86 3′UTR positive FeCV samples and 20/49 3′UTR positive FIPV samples (Figure 3). The M1030 and S1032 loci were analyzed for the presence of mutations. None of the 35 FeCV samples had a mutation at either location. In the samples derived from FIP-positive cats, however, 8/20 of the S-protein gene regions sequenced had a M1030L mutation and 1/20 had an S1032A mutation. In 3/20 FIP samples and 1/35 FeCV samples, a T1034S mutation was detected, whereas 8/20 FIP samples had no mutation at any of these locations. One other locus, D1014, could be identified that contained several different mutations in both FIPV and FeCV samples, including D1014G (n = 2), D1014A (n = 3), D1014S (n = 3), and D1014Q (n = 3).

Discussion

Feline coronavirus type 1 was the only serotype we could detect in the fecal and tissue samples collected. This is consistent with previous studies that found FeCV type 1 to be the most commonly isolated serotype in North American and European populations of cats (4,7,10). Our observed number of positive cats (46%) is comparable to the results of previous reports (1,10). We expected a higher prevalence of FeCV-infected cats in shelters than in household cats. Interestingly, this was not the case, which may be explained by the high proportion of household cats that were either originally obtained from a rescue or were from multi-cat households in our sample. Additionally, we collected feces on intake to the shelter before the cat could be infected with strains circulating in the shelter. It has consistently been found that FeCV infection is significantly more prevalent in multi-cat households, catteries, and shelters (10,15).

While the FIP virus is a mutated variant of FeCV, it is not yet fully understood how this mutation arises or where it is located in the genome. Phylogenetic analysis of our FeCV and FIPV samples showed no clustering due to the geographic origin or FeCV *versus* FIPV infection. This supports the internal mutation theory of FIP infection that is favored by most researchers. In this theory, the mutation causing FIP arises *de novo* in each cat and cannot be transmitted (15,16). The alternative theory postulates that circulating virulent virus (FIP) and non-virulent strains (FeCV) are present (17), although we were unable to detect a separate FIPV lineage in FIP cats in this study.

We carried out a mutation analysis on the specific region of the S-protein gene targeted by the IDEXX FIP Virus RealPCR test (9). Chang et al (5) reported that $>$ 95% of FIP-positive cats in Europe could be identified by mutations in 1 of 2 amino acids (M1030L or S1032A). Lewis et al (6) also reported the M1030L mutation in all 3 of the FIP genomes they sequenced. In contrast, we found that only 40% of FIP-positive cats in this study had the M1030L mutation and only 1 out of 20 FIP-derived sequences had the S1032A mutation. In our samples, 40% had no mutations in this region, while 15% had a T1034S mutation. We expected the majority of our FIP samples to have 1 or both mutations. Five of these no-mutation samples may have contained intestinal tissue and we could have amplified wildtype FeCV. Six pathological samples did not contain intestines, however, and did not have either mutation present. Another explanation could be that the virus presents itself as a quasispecies and we amplified the non-mutated form of the virus (18). Next-generation sequencing would be required to answer this question. A third option is that FIPV found in western Canada may have additional or alternative virulence sites that have not yet been identified. It has been postulated that the furin cleavage site within the S protein is a possible virulence region (7), which would be worthwhile investigating. If this is the case, caution would be advised in interpreting negative results on IDEXX's FIP Virus RealPCR test.

While there was a region of increased variability in both FeCV and FIP samples at position D1014 of the S-protein genome, these were all conservative mutations that resulted in amino acids with similar biochemical properties, which are unlikely to change the functionality, structure, or antigenicity of the S-protein.

In our study, 45% of the FIP viruses derived from clinical cases in western Canada contained 1 of the previously described M1030L or S1032A mutations. While this supports the idea that these mutations play an important role in the virulence of FIPV, it suggests that a large proportion of FIPV is lacking either mutation.

Acknowledgments

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